Identification and Characterization of Layer-Specific Differences in Extraocular Muscle M-Bands

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PURPOSE. To examine and characterize the expression of M-bands (or M-lines) in the orbital layer (OL) and global layer (GL) of adult rat extraocular muscles (EOMs).

METHODS. Semiquantitative polymerase chain reaction (PCR), quantitative (q)PCR, immunohistochemistry, and confocal microscopy were used to analyze expression of the major gene and protein constituents of M-bands in freshly dissected and cryosectioned rectus extraocular muscles (EOMs) and tibialis anterior (TA) muscles. Electron microscopy (EM) was performed on perfusion-fixed EOMs and TA muscles in a layer-specific manner, to determine, characterize, and quantify laminar-specific differences in M-band expression.

RESULTS. These studies demonstrate EOM layer-specific differences in the expression of M-bands and their major constituents, myomesin1 (Myom1) and myomesin2 (Myom2 or M-protein) at the structural, mRNA, and protein levels by using EM, semiquantitative PCR, qPCR, immunohistochemistry, and confocal microscopy. Differences in thick filament lattice order were quantified by using EM-based inter-thick-filament distance and variance measurements and were found to be TA > GL > OL.

CONCLUSIONS. The expression pattern of M-bands and their constituents in EOMs provides mechanistic insight for their allotopic and layer-specific viscoelastic properties. Modeling the differential expression of M-bands between EOMs and TA predicts increased elasticity but reduced force and eccentric contraction (ECC)–mediated damage in EOMs and suggests a potential mechanism for the clinical sparing of EOMs noted in Duchenne’s muscular dystrophy (DMD). (Invest Ophthalmol Vis Sci. 2007;48:1119–1127) DOI:10.1167/iovs.06-0701

Mammalian EOMs are a highly specialized group of skeletal muscles that form part of the oculomotor system. They are organized into two functionally distinct layers: an outer orbital layer (OL) and inner global layer (GL). EOMs exhibit a number of fundamental differences compared with other skeletal muscles. Indeed, the differences are so marked that the term allototype has been suggested to define a unique, functional niche for these muscles. We and others have demonstrated that EOMs have a unique, molecular allototype as well in rodents and in humans. One of the most intriguing consequences of the allototype is their differential response to disease, as exemplified by their preferential involvement in Duchenne muscular dystrophy (DMD), despite the severe, widespread necrosis in other skeletal muscles in this disease.

Basic mechanical properties of muscle such as contractility and elasticity are thought to depend largely on the fundamental unit of muscle organization, the sarcomere. The M-band, situated in the center of the sarcomere, has been suggested to be crucial for stability of sarcomeric contractions. Ultrastructurally, the M-band can be resolved as a variable number of parallel lines in longitudinal sections; these thin lines can also be seen connecting adjacent thick filaments with one another in cross sections, where they are also referred to as M-bridges (reviewed in Ref. 23) Recent work suggests that rather than a rigid structure, the M-band is an elastic web cross-linking thick filaments. Important constituents of M-bands include Myom1, Myom2, and creatine kinase (CK-MM). In certain muscle groups, alternative splicing of the Myom1 mRNA leads to the expression of an approximately 100-amino-acid longer splice variant known as EH-myomesin. Myomesin is considered to be the principal thick filament cross-linking protein, and its overall content (i.e., sum of EH and non-EH isoforms of Myom1) seems to be roughly consistent in heart, limb, and eye muscles of mice.

Whereas a general consensus exists on M-band appearance in limb muscle, there are several open questions regarding M-band appearance in EOMs. To address these questions, we studied M-bands at the mRNA, protein, and structural levels using a variety of molecular and structural methods including semiquantitative polymerase chain reaction (PCR), quantitative (q)PCR, and light and confocal immunohistochemical microscopy as well as electron microscopy (EM). Care was taken in the experimental design to take into account the complex three-dimensional anatomy of EOMs while performing our detailed characterization of M-bands in EOMs and tibialis anterior (TA) muscles.

MATERIALS AND METHODS

RNA Isolation, Semiquantitative PCR, and qPCR

Animals were maintained in approved accommodations at the University of Pennsylvania and were used in accordance with the ARVO Statement for the Use of Animals in Ophthalmic and Vision Research.

Two adult rats (Sprague-Dawley, >250 g) were killed by CO 2 asphyxiation. Dissection of the EOMs and TA, RNA processing, semiquantitative PCR, and qPCR were performed as previously described. PCR conditions were denaturation at 94°C for 4 minutes, followed by 30 seconds each at 94°C and 55°C, and extension at 72°C

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Supported by National Eye Institute Grant EY013862.

Submitted for publication June 23, 2006; revised September 18 and November 15, 2006; accepted January 19, 2007.

Disclosure: M.H.J. Wiesen, None; S. Bogdanovich, None; I. Agarkova, None; J.-C. Perriard, None; T.S. Khurana, None

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for 90 seconds. To ascertain overall Myom1 content, we designed primers in the 3' untranslated region, detecting both non-EH and EH-containing splice forms. Rat Myom1 (NCBI XM_237525) primers were (5'-3'): forward GACCTCAAGGTAGGTCATC; reverse GGATCTCCTATCTCTAAACGATC. For quantifying relative expression of non-EH and EH-containing splice forms the primers used were (5'-3'): forward GCGAATTACCATCCAAATG; reverse ATAAATAGGCCTGTCAGG. Rat Myom2 (NCBI XM_240481) primers were (5'-3'): forward CAGTCTGCTGTCATCG; reverse CGGTGATT- GTCTACGGTTGAAG. Rat CK-MM (NCBI NM_017008) primers were (5'-3'): forward GACAAATCATCCCAAGTAG; reverse ATAATAGCCCTAACCAGAAGAC. Rat Myom2 (NCBI XM_237523) primers were (5'-3'): forward CGCATGTCGGTCCATCTC; reverse GCAAAGTGTGATGACC.

Tissue Preparation for Light Microscopy and EM

Methods used for light microscopy and EM were as previously described. To ensure adequate fixation, the left common carotid and right external iliac arteries were cannulated with 0.8-mm diameter tubing and rats perfused with 0.1 M Ca2+/Mg2+-free PBS for 1 minute to relax musculature, followed by fixation using 6% glutaraldehyde in 0.1 M cacodylate buffer for 20 minutes at a flow rate of 2.5 mL/min. Muscles were removed en bloc, postfixed with 2% OsO4 for 1 hour, and embedded in Epon.

Light Microscopy

Semithin cross-sections were cut from whole recti muscles (Ultratrac UCT microtome; Leica, Vienna, Austria). Photomontages of the entire rectus muscle cross section were created digitally (Photoshop ver. 7.0; Adobe Systems, San Jose, CA) and served as a map for electron microscopy.

Electron Microscopy and Morphometric Analysis

Transverse sections of entire recti EOMs were used to score OL and GL fibers separately for M-bands. In each of three independent preparations, 100 OL, 100 GL, and 100 TA fibers were analyzed. For longitudinal sections, the recti muscle blocks underwent re-embedding, and ultrathin sections of both layers were cut. Morphometric analysis was performed using ImageJ software (available by ftp at zippy.nimh.nih.gov or at http://rsb.info.nih.gov/nih-image; developed by Wayne Rasband, National Institutes of Health, Bethesda, MD). A total of 1440 inter–thick-filament measurements were performed in the M-band region in 24 randomly chosen fibers (six each) out of the OL, GL, large pale GI, and TA, with 60 measurements being performed in each fiber.

Immunofluorescent and Confocal Microscopy

Previously described methods from our laboratories were used to study two independent preparations. Ten-micrometer-thick frozen sections were incubated with mouse monoclonal antibodies α-all Myom1, clone B4; rabbit polyclonal antibodies α-EH myomesin25; and mouse monoclonal antibodies α-Myom2, clone AA25937 (generously donated by Dieter Först, University of Bonn), washed, incubated with appropriate secondary antibodies, and imaged.

RESULTS

Expression of M-Band Constituent mRNAs in EOMs and TA

Two independent assays were used to determine expression levels of genes encoding major M-band constituents Myom1, Myom2, and CK-MM. Semiquantitative PCR showed that mRNA levels for Myom1 and CK-MM were mildly (less than twofold) decreased, whereas expression of Myom2 was markedly reduced in EOMs compared with TA (Fig. 1A). Samples were independently analyzed using qPCR, and concordant and com-
parable changes were obtained as well (Fig. 1B). These data were also consistent with our previous expression-profiling data comparing limb and EOMs, where only Myom2 was found to be reduced (by 21-fold) in EOMs, when a twofold difference cutoff was used.17 In addition, quantification of EH and non-EH isoforms of Myom1 (Fig. 1C), confirmed that the EH-myomesin splice form was the predominant isoform of Myom1 expressed in EOM29,30 and that it was not detectable in TA (Supplementary Fig. S1, http://www.iovs.org/cgi/content/full/48/3/1119/DC1), as previously described.25

**Electron Microscopic Characterization of M-Bands in Longitudinal Sections**

Having shown that EOMs and TA express mRNAs encoding major M-band constituents and thus have the capacity to form M-bands, we examined longitudinal sections of EOMs by EM to determine whether M-bands could be visualized in EOMs. Given the contradictory reports of the presence and absence of M-bands in the literature, we believed it was critical to obtain unambiguous, layer-specific tissue from EOMs to address possible laminar differences in expression of these important structures. As shown in the photomontages in Figure 2, an entire EOM rectus was embedded, and semithin sections were cut and visualized using light microscopy to orient the EOM block unambiguously, with respect to the OL and GL (Fig. 2A). The block was rotated 90° across its long axis and re-embedded to obtain OL- and GL-specific tissue at the planes shown, rotated back to its original orientation, and re-embedded to obtain adjacent sections and confirm that only OL- and GL-specific tissue had been captured using this procedure (Fig. 2B; arrowheads). The entire procedure was repeated to obtain images from the middle of the GL (Fig. 2B, 2C; arrowheads). The layer-specific tissue-containing blocks were individually re-embedded to allow us to cut longitudinal sections and examine OL- and GL-specific tissue. As shown in Figure 2D, sections obtained from OL showed a paucity of distinct M-
bands. Examination of sections from GL-specific tissue (Figs. 2E, 2F) revealed well-formed M-bands in some sarcomeres, suggesting that success in visualizing M-bands on EM in longitudinal sections may depend on which layer of the EOM is sampled. The entire procedure was repeated on a different rectus from the same animal (Figs. 2A’–2F’), as well as in a rectus from a different animal (Supplementary Fig. S2, http://www.iovs.org/cgi/content/full/48/3/1119/DC1).

Detailed Characterization of M-Bands in Transverse Sections

The curvature and complicated anatomic relationship of the EOM layers33,34 obviates the possibility of comprehensively studying M-bands using longitudinal sections. Hence, to confirm and extend the presence and distribution of M-bands noted using longitudinal sections, we decided to study them in transverse sections by EM. The basis of this strategy is outlined in Figure 3. Figure 3A shows the classic appearance of M-bands in longitudinal sections, and Figure 3B shows a sketch labeling components of the sarcomere. Figure 3C depicts a projection of thick filaments and M-band structures that would be obtained by viewing a longitudinal section cut in the middle of the H-zone and rotated 90° in space along its long axis: where M-bands can be identified as fine lines or M-bridges,38 connecting thick filaments with one another. Figure 3D is an actual EM showing the M-band and thick filaments obtained by making a transverse section visualized at the middle of the H-zone. The incomplete register of individual sarcomeres in myofibrils within the thickness of sectioned material allows visualization of A and I bands (including the H-zone) in most of the sections and hence allows one to score presence or absence of M-bands in different myofibers.26 Although this strategy is more labor intensive, it affords the possibility of comprehensively and accurately studying the distribution of these specializations in EOMs.

Using this strategy, we were able to visualize unambiguously the EOM fibers from the OL and GL separately, to identify and study the M-bands in anatomically and physiologically distinct EOM fiber types. M-bands were clearly visible in cross sections of TA (Figs. 4A, 4A’) and also in cross-sections of GL (Figs. 4B–D, 4B’–D’). However, variations were noted in their appearance and extent among the different fibers. GL fibers with high sarcoplasmic reticulum (SR) content combined with fewer mitochondria (Figs. 4B, 4B’) exhibited M-bands similar to those seen in TA. Considering the M-band appearance, fiber size, mitochondrial, and SR content, we identified these fibers as the previously described large pale fibers of the GL.4 However, M-bands were found to be less well-formed in other GL-fibers such as those containing relatively little SR content combined with low numbers of mitochondria and those containing fairly high SR and high mitochondrial content (Figs. 4C, 4D’). The extent of the variation between muscle groups (i.e., between the TA and EOMs), as well as the variation within a group (i.e., within different layers of EOM), was quantified by scoring 900 randomly chosen fibers for the presence or absence of distinct M-bands (Fig. 5).

Having demonstrated differences in M-band appearance between different muscles as well as between different layers of the same muscle, we addressed the functional consequence of differences in M-band composition in the organization of thick filament lattice by measuring distances between thick filaments (to quantify lattice order). Thus, distances between adjacent thick filaments in randomly chosen TA, OL, GL, and large pale GL fibers were measured. TA fibers had a highly ordered lattice structure, OL fibers had poorly ordered lattices, and fibers from GL and large pale GL fell between the TA and OL (Fig. 6).

Spatial Variation in Distribution of M-Band Constituent Proteins in TA and EOMs

To validate these findings independently and address the spatial distribution of M-band constituents, we examined frozen
sections by immunofluorescence microscopy. The α-all-Myom1 antibody detects known isoforms of Myom1, the EH-myomesin antibody detects only the EH splice variant of Myom1, and the Myom2 antibody detects known isoforms of Myom2. Figure 7A (left) shows that TA was evenly labeled with both the α-all-Myom1 (top row) and Myom2 (middle row) but not significantly labeled with the EH-myomesin (bottom row) antibodies. In EOMs (middle column higher magnification, right column lower magnification), all fibers were labeled with α-all-Myom1 antibodies, with slightly greater signal in the OL. Consistent with our mRNA data, the major structural constituents of M-bands are present in EOMs at the protein level. To further define the complex layer-specific expression pattern, we performed double labeling using the EH-myomesin and the Myom2 antibodies. As shown in Figure 7B, this combination labeled both the OL and GL, with EH-myomesin being predominantly expressed in OL (Fig. 7B, left column), whereas Myom2 labeling restricted to the GL (Fig. 7B, middle column). The reciprocal expression pattern of these antibodies (Fig. 7B, middle column) also extended to the GL, where apart from the reciprocity observed in the OL, the GL fibers were noted to express either EH-myomesin or Myom2, but not both together. A subset of GL fibers was not labeled with either antibodies. Thus, EH-myomesin expression was detectable in EOMs and not in the TA. In contrast, the Myom2 antibodies labeled only ~30% of fibers in the GL, whereas the OL was mainly negative for Myom2. Conversely, the EH-myomesin antibodies evenly labeled OL-fibers but only a few fibers with strong labeling for EH-myomesin were detected in the GL.

To extend and confirm these results, we performed confocal microscopy of whole mount preparations of EOMs in longitudinal optical planes. As shown in Figures 7C and 7D, confocal microscopy revealed a similar pattern of labeling and unequivocally demonstrates M-bands that are seen to occur in a regular pattern of repeating transverse lines. The α-all-Myom1 antibodies labeled M-bands in both OL and GL fibers, EH-myomesin predominantly labeled OL fibers, whereas Myom2 labeling was restricted to some GL fibers. Using this sensitive method, we detected complex patterns of myomesin expression: We demonstrated that some fibers coexpressed both EH-myomesin and Myom2, even with one of the constituents being expressed only at minor levels. Fiber D II strongly expressed Myom2 with traces of EH-myomesin.

**DISCUSSION**

We used a combination of molecular and ultrastructural methods to perform a detailed characterization of M-bands and their constituents in adult rodent EOMs and TA limb muscles. Molecular studies demonstrated that both express mRNA encoding M-band constituents and thus have the capacity to form M-bands (Fig. 1). At the ultrastructural level, we were able to demonstrate distinct M-bands both in longitudinal sections (Fig. 2) as well in transverse sections of TA and EOM fibers (Figs. 3, 4). Considerable inter- and intralayer variability in M-band expression and lattice structure order were noted in EOMs (Figs. 4, 5, 6). Immunohistochemical analysis provided spatial information regarding the expression of M-band constit-
uents and independently validated our mRNA and ultrastructural studies at the protein level (Fig. 7). Finally, we propose a model predicting functional consequences of differential expression of M-bands in TA and EOM (Fig. 8).

The fiber- and layer-specific differential expression of M-bands coupled with the complex, highly contoured, and layered anatomy of EOMs may offer an explanation for the differences noted in the literature.\(^4,27-30\) Thus, if our analysis had been limited to examining longitudinal OL sections (Fig. 2B) rather than a detailed analysis of both OL and GL (Fig. 2A) as well as examining different fibers within each layer (Figs. 4, 7), we would have concluded that M-bands and their constituents were missing in adult rodent EOMs. In accordance with a previous study by Mayr,\(^4\) the large pale fibers of the GL (consisting \(\sim 30\%\) of the GL) had the most distinct M-bands on EM, comparable to M-bands seen in the TA (Fig. 4). Myom2 labeling exhibited restricted expression and was noted in approximately the same percentage of GL fibers (Fig. 7A), suggesting the intriguing possibility that the large pale GL fibers were the ones that were labeled by Myom2 antibodies (Fig. 6). The OL was essentially negative for Myom2 and had the poorest lattice order (Fig. 6). The OL was less distinct in M-bands compared with the complex, highly contoured, and layered anatomy of EOMs.\(^5,27-30\) Thus, if our analysis had been limited to examining longitudinal OL sections (Fig. 2B) rather than a detailed analysis of both OL and GL (Fig. 2A) as well as examining different fibers within each layer (Figs. 4, 7), we would have concluded that M-bands and their constituents were missing in adult rodent EOMs. In accordance with a previous study by Mayr,\(^4\) the large pale fibers of the GL (consisting \(\sim 30\%\) of the GL) had the most distinct M-bands on EM, comparable to M-bands seen in the TA (Fig. 4). Myom2 labeling exhibited restricted expression and was noted in approximately the same percentage of GL fibers (Fig. 7A), suggesting the intriguing possibility that the large pale GL fibers were the ones that were labeled by Myom2 antibodies (Fig. 6). The OL was essentially negative for Myom2 and had the poorest lattice order (Fig. 6). Independent support for these layer-specific differences in expression of Myom1 and -2 also comes from our previous laser capture microscopy-based expression profiling study where we found Myom1 was expressed at \(\sim 1.8\)-fold higher levels in the OL. Conversely, we found an \(\sim 18\)-fold downregulation of Myom2 mRNA in the OL compared with GL in adult rat EOMs.\(^39\) These data also support the idea of layer-specific functional roles in eye movements.\(^5\)

We believe that a functional consequence of the layer- and fiber-specific differences in individual constituents of M-bands is the generation of poor lattice order in fibers with less distinct M-bridges (Figs. 6, 8) leading to the appearance of "fuzzy" sarcomeres\(^22\) or sarcomeres characterized by broader Z-lines, variable length of thin filaments and less distinct M-bands in longitudinal sections (compare Figs. 2D, 2D' with 2E, 2F and 2E', 2F'). Fibers with weaker M-bands combined with broader Z-lines in longitudinal sections and indistinct M-bridges in transverse sections have been seen in a subset of soleus fibers,\(^39\) have been correlated with the expression of EH-myomesin and the downregulation of Myom2, and have been proposed to be an adaptation to improve resistance under ECC conditions.\(^32,23\) Indeed, single-molecule measurements suggest that EH-myomesin is mostly unfolded and functions as an entropic spring in the middle of the myomesin molecule analogous to the PEVK region of titin.\(^60\) Conversely, Myom2 is thought to improve the stability of the thick filament lattice of fast fibers by increasing their stiffness.\(^41\)

Our model predicts that fibers such as those in the EOMs (in particular OL fibers that express EH-myomesin but lack...
Myom2) would generate less force but be more elastic and resistant to ECC damage (Fig. 8). We would predict that OL fibers would be more elastic than GL fibers; which in turn would be much more elastic than TA fibers. These mechanical properties also offer an advantage to resisting damage in DMD, where, because of the genetic absence of dystrophin,

Myom2 antibodies did not label OL fibers (with few exceptions). (A, bottom row): α-EH-myomesin antibodies did not label TA fibers but strongly labeled OL fibers. Conversely, only some GL fibers showed strong labeling. (B) Double-staining of adult rat EOMs using α-EH-myomesin and α-Myom2 antibodies: α-EH-myomesin antibodies (red) strongly labeled fibers in the OL and only some fibers in the GL. α-Myom2 antibodies (green) significantly labeled ~30% fibers in the GL, but OL fibers were negative (with few exceptions). The remaining GL fibers were negative for both antibodies. (C, D) The fiber- and layer-specific, reciprocal expression of EH-myomesin and Myom2 in longitudinal sections of EOMs examined by confocal microscopy. (C) OL fibers and (D) GL fibers. α-Myom1 antibodies evenly labeled fibers in both layers of EOMs. Fibers in the OL were positive for EH-myomesin and negative for Myom2 (C). GL fibers (D) showed a fiber-specific reciprocal expression of EH-myomesin and Myom2. Fiber D II showed little labeling for EH-myomesin but was strongly positive for Myom2, while D III shows a reciprocal pattern of labeling. Occasionally, fibers basically negative for both EH-myomesin and Myom2 (D V) could also be visualized. Scale bar: (A, left and middle columns; B) 150 μm; (A, right column) 300 μm; (C, D) 10 μm.

Note, the soleus muscles of the mdx mouse (animal model of DMD) have fuzzy sarcomeres and are more resistant to ECC-induced damage than are other skeletal muscles. Moreover, the utrophin/dystrophin double-knockout model shows layer-specific differences of EOM involvement. Although the predictions of this model need to be tested to validate them, it is interesting to point out that consistent with our model, direct measurements of canine rectus muscle strips revealed lower elastic and greater viscous compo-
safeguard for sarcomere stability?


